This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.14461
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Accepted Article

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Abstract
Tropical mountains are areas of high species richness and endemism. Two historical phenomena may have contributed to this: (1) fragmentation and isolation of habitats may have promoted the genetic differentiation of populations and increased the possibility of allopatric divergence and speciation, and; (2) the mountain areas may have allowed long-term population persistence during global climate fluctuations. These two phenomena have been studied using either species occurrence data or estimating species divergence times. However, only few studies have used intraspecific genetic data to analyse the mechanisms by which endemism may emerge at the microevolutionary scale. Here, we use landscape analysis of genomic SNP data sampled from two high-elevation plant species from an archipelago of tropical sky-islands (the Transmexican Volcanic Belt) to test for population genetic differentiation, synchronous demographic changes and habitat persistence. We show that genetic differentiation can be explained by the degree of glacial habitat connectivity among mountains, and that mountains have facilitated the persistence of populations throughout glacial/interglacial cycles. Our results support the ongoing role of tropical mountains as cradles for biodiversity by uncovering cryptic differentiation and limits to gene flow.

Introduction
Tropical mountains are biodiversity hotspots that tend to have more species than the lowlands surrounding them (Myers et al. 2000). Their levels of species richness are particularly high due to both the presence of species with broad distributions and aggregations of locally endemic species (Kruckeberg & Rabinowitz 1985; Jetz et al. 2002; Linn et al. 2019).
Variation in contemporary environment may provide some explanation for regional variation in the richness of geographically wide-ranging species, but the high level of endemism present in tropical mountains exceeds predictions using macro-ecological variables alone (Jetz & Rahbek 2002; Rahbek et al. 2007). This excess may, however, be explained if analyses incorporate the history of species and their habitats (Jetz et al. 2004; Graham et al. 2006; Fjeldså et al. 2012). Such an integrative approach has lead to the suggestion that tropical mountains are rich in biodiversity because they promote both species differentiation and long-term (glacial-interglacial) population persistence (Fjeldså et al. 2012). This represents a new and exciting advance that calls for phylogenetic and phylogeographic data to further our understanding of the origin and maintenance of low-latitude mountain biodiversity.

The small surface area of high mountain regions and their geographic isolation leads to small and fragmented populations. Such a scenario is expected to favour allopatric speciation and hence the evolution of many new endemic taxa (Kessler 2002). Several studies confirm this expectation and it is the most commonly cited explanation for elevational patterns of endemism (Kessler 2002). Parapatric speciation may also occur, although it seems to be a less frequent phenomenon (Weir 2009; Cadena et al. 2011; Päckert et al. 2012). With regard to allowing population persistence through time, tropical mountains have been found to be areas of low climate change velocity, meaning they are areas where biodiversity can survive through periods of global climate fluctuation through small altitudinal shifts instead of long latitudinal movements (Loarie et al. 2009; Sandel et al. 2011). Areas of low climate change velocity thus allow for relatively long-term (glacial-interglacial) in-situ (within a close horizontal distance to where they exist today) population persistence, in contrast to the range shifts or extinctions that are inferred for higher latitudes and shallower orography (Hewitt 1996;
Population persistence is meaningful for the accumulation of endemism because it leads to the local aggregation of endemic species over time (Fjeldså et al. 1999).

The allopatric speciation and long-term persistence hypotheses have been examined using species occurrence data (e.g. Sandel et al. 2011; Krömer et al. 2013) and, more recently, incorporating molecular data for the estimation of species divergence times (e.g. Smith et al. 2014). The scale of analysis ranges from coarse continental data (Rahbek et al. 2007; Sandel et al. 2011; Fjeldså et al. 2012) to more detailed analyses of specific mountain ranges such as the Andes (e.g. Fjeldså et al. 1999; Kessler 2002), the Himalayas (e.g. Päckert et al. 2012) and the Eastern Arc Mountains of Tanzania and Kenya (Fjeldså & Bowie 2008). Although these studies have included an evolutionary perspective by jointly analysing species ranges with phylogenetic data, there remains a need for intraspecific analyses of the mechanisms by which endemism may emerge at the microevolutionary scale.

We address this knowledge gap by examining population genetic differentiation and habitat persistence within high-altitude tropical mountains, using as a study area the highest mountains of the Trans-Mexican Volcanic Belt (TMVB, Fig. 1). We take a population level approach because it is expected that areas that facilitate population persistence over intraspecific (phylogeographic) timescales should, in the absence of further geological change, also be stable across interspecific (phylogenetic) timescales, such that regions of genetic endemism will eventually lead to regions of high species diversity (Hugall et al. 2002; Carnaval et al. 2009). Thus, examining population differentiation and long-term population persistence at the microevolutionary scale can contribute to the evolutionary understanding of tropical mountain biodiversity.
To analyse tropical montane taxa over a timescale consistent with population genetic process, we frame our hypotheses under the expectations of a sky-island dynamic for the TMVB (Toledo 1982; Mastretta-Yanes 2015a). The TMVB is an area that comprises an archipelago of sky-islands at ~19ºN where the highest stratovolcanoes (>3,000 masl) emerged during the last 1.5 Myr (Ferrari et al. 2012). Species with current range limits above 3,000 masl are expected to: (1) have been restricted to high-elevation refugia during the interglacial periods of the Pleistocene (such as the present), where divergence could be promoted by restricted gene flow; and (2) have extended ephemerally to lowlands during glacial periods, where the probability of genetic admixture would be increased (Toledo 1982; Mastretta-Yanes 2015a). Therefore, for the TVMB, population differentiation is expected to be a function of topographical variables. Demographically, this sky-island dynamic translates into cycles of population contraction (interglacial periods) and expansions (glacial periods), which should be temporally synchronous among the different mountains, assuming these climatic conditions resulted in a pervasive effect. If glacial-interglacial habitat persistence occurred within mountains that are isolated from each other over extended periods, populations would be expected to accumulate private genetic variation.

To test the above-mentioned expectations, we focus on two timberline-alpine grassland plant species of the TMVB for which we generated genomic SNP data, *Juniperus monticola* and *Berberis alpina*, and the glacial/interglacial distribution of their habitat type. We first examine the distribution of suitable conditions across sampled mountains through limited altitudinal range changes during glacial/interglacial stages. We then investigate if genetic differentiation can be explained by the degree of historical or contemporary habitat connectivity among mountains. Finally, we assess co-demographic change across populations within a comparative framework. We then use
these data to evaluate the general hypothesis that long-term environmentally stable conditions for these ecosystems persisted in situ throughout glacial/interglacial cycles.

Methods

Study system and sampling

Juniperus monticola and Berberis alpina were chosen for analysis from an initial set of five species (including Eryngium proteiflorum, Cirsium ehrenbergii and Pinus hartwegii) that have broad distributions within the TMVB and are easily distinguished from related species in the field (Mastretta-Yanes 2014). Eryngium proteiflorum and C. ehrenbergii were not chosen for analysis because they are not diploid, and J. monticola and B. alpina were chosen over P. hartwegii because the latter also extends to Northern Mexico and Central America. Juniperus monticola and B. alpina are both shrubs that grow from 3,300 to 4,200 metres above sea level (masl) on rocky formations from above the timberline and into alpine grasslands of the TMVB (Adams 2014; Mastretta-Yanes 2014). Both species can live from several decades to up to few hundreds of years (Francis 2004; Adams 2008). Their reproductive activity starts at an age of 4-7 years in the case of Berberis (Peterson 2003) and of 10-20 years in the case of Juniperus (Bonner 2008), with both species showing irregular heavy seed crops and seed dormancy (Francis 2004; Bonner 2008). Regarding dispersion, both species produce fleshy fruits and cones, which in other closely related species are dispersed by birds and small mammals (Adams 2008; Peterson 2003). Thus, while these species are not phylogenetically close at all, they have relatively similar life histories and dispersal abilities. They are closely related to J. flaccida and B. moranensis, which grow at lower altitudes (800-2,600 masl and 1,800-3,150 respectively).
Mountain peaks from >3,000 masl within the TMVB and nearby areas of the Altiplano Sur (AS) and the Sierra Madre Oriental (SMOr) were surveyed for *B. alpina* and *J. monticola* during September-October 2010 and April-May 2011 (Fig. 1). *Berberis alpina* was found in a total of six of the 17 locations surveyed, and *J. monticola* in 13, which represent their known distribution within the TMVB and the AS. Samples of the closely related species and outgroups *B. moranensis, B. trifolia, B. pallida, J. flaccida, J. zanonii* and *J. deppeana* were collected at lower elevations (~2,000-3,150 masl for *Berberis* and ~800-2,500 masl for *Juniperus*) of the TMVB and at northernmost localities of the SMOr and Sierra Madre Occidental (SMOcOc) in October 2010 and 2012. Sampling was performed with SEMARNAT permission No. SGPA/DGGFS/712/2896/10. Herbarium specimens of *B. alpina, B. moranensis, J. flaccida* and *J. monticola* were prepared and deposited within the Herbario Nacional in Mexico City (MEXU) or within Herbario CIIDIR in Durango.

**Molecular methods**

Based on data from related species, the sampled *Berberis* species have an expected genome size between 0.50 and 1.83 Gbp (Rounsaville and Ranney, 2010), while the range is from 9-10 Gbp for the *Juniperus* species (Zonneveld 2012). For both taxa, ddRAD libraries were prepared using modified versions of protocols by Parchman et al. (2012) and Peterson et al. (2012). For *Berberis*, the enzyme pair EcoRI-HF and MseI was used, while for *Juniperus*, the rare cutter SbfI-HF was used instead of EcoRI-HF, thus allowing for a narrower subsampling of the larger Juniper genome. Samples were randomly divided into three (*Berberis*) or 10 (*Juniperus*) groups with a common sequencing index (ddRAD libraries hereafter). All *Berberis* and two *Juniperus* libraries were sequenced using single-end reads (100 bp long) in a separate lane of an Illumina
HiSeq2000, while two libraries were sequenced in a single lane for the rest of the Juniperus libraries. Further details on Berberis laboratory protocol and sequencing output are detailed in Mastretta-Yanes et al. (2015b). For Juniperus this information is available in Supporting Information 1.

The Berberis dataset consists of 75 individually tagged specimens of B. alpina and B. moranensis (6-10 per mountain, Table 1), three samples of each outgroup (B. trifolia and B. pallida) and 15 replicated samples, with at least one replicate per population or species. The Juniperus dataset consists of 137 individually tagged specimens of J. monticola (10 per mountain, Table 1), four of J. flaccida, one of J. deppeana, one of J. zanonii, 10 negative controls and 20 replicated samples, with at least one replicate per sampling locality or species (with the exception of J. deppeana).

Sequencing output, de novo assembly and loci filtering of RAD data

Complete details of Berberis sequencing output and quality filtering are available in Mastretta-Yanes et al. (2015c). Briefly, after demultiplexing and quality trimming of Berberis raw reads, final sequences were 84 bp long. Juniperus raw reads were demultiplexed and quality filtered using Stacks v. 1.17 by: (1) truncating final read length to 87; (2) removing any reads with an uncalled base; (3) discarding reads with low quality scores (score limit 22 to 28, depending on the library); (4) discarding reads that have been marked by Illumina’s chastity filter as failing; (5) filtering adapter sequences, and; (6) rescuing tags (allowing a mismatch of one between barcodes). See Supporting Information 1 for full details on Juniperus bioinformatic pipeline.

Here we refer to a RAD-locus as a short DNA sequence produced by clustering together RAD-alleles; in turn, RAD-alleles differ from each other by a small number of SNPs in certain nucleotide positions (SNP-loci). Data were de novo assembled using the
software Stacks (Catchen et al. 2011, 2013). Data from Berberis had been previously assembled in Stacks v. 1.02 with the parameter values $m=3$, $M=2$, $N=4$, $n=3$, $\text{max\_locus\_stacks}=3$ and a SNP calling model with an upper bound of 0.05 (Mastretta-Yanes et al. 2015c). Stacks v. 1.17 was used for Juniperus with the parameter values $m=10$, $M=2$, $N=4$, $n=3$, $\text{max\_locus\_stacks}=4$ and default SNP calling model. These settings were chosen after testing a wide range of parameters as in Mastretta-Yanes et al. (2015c), and optimising the recovery of a large number of loci while reducing the SNP and RAD allele error rates (Supporting Information 1). After de novo assembly, the data were filtered to keep only those samples that had more than 50% and 35% of the mean number of loci per sample for Berberis and Juniperus, respectively (different percentages were used according to sample size), and only those loci present in at least 80% of Berberis samples and 70% of Juniperus. Putative paralogous loci of the Berberis dataset were filtered by identifying loci where the frequency of the major allele equalled $p=0.5$ in more than one population or species, as detailed in Mastretta-Yanes et al. (2014). For the Juniperus dataset the same procedure was followed, but with the following modifications in response to the larger sample size: (1) putative paralogous loci had to meet the extra condition of showing the deviations from Hardy-Weinberg Equilibrium (HWE) of $H_{\text{obs}}>0.9$, negative $F_{\text{IS}}$ or $F_{\text{IS}}=1$, and (2) putative paralogous loci private to a single population of J. monticola were also excluded by identifying loci where $p=0.5$ in any single sampling location, present in more than three individuals of that population and showing deviations from HWE. To ameliorate the effect of missing data on population genetics statistics, RAD-loci that were present in several sampling locations but represented by only one individual in any given population were also filtered. These extra conditions were not performed in the Berberis dataset due to the small sample sizes for some locations. Replicates were used to estimate error rates for
both taxa as in Mastretta-Yanes et al. (2015c). For downstream analyses, only one sample for each replicate pair was used, along with all the remaining non-replicated samples. For both species, only the first SNP of each RAD-locus was used for the population genomic analyses described below (to control for linkage disequilibrium).

Considerably fewer loci were recovered in *Berberis pallida*, compared to the other *Berberis* species, which is likely explained by mutations affecting restriction enzyme cutting sites as a consequence of a distant evolutionary relationship with the other species in the study. This species was therefore excluded from further analyses.

*Population genomic statistics and population differentiation*

The *populations* program of *Stacks* was used to estimate the number of private alleles, the percentage of polymorphic loci, heterozygosity, $\pi$, and $F_{IS}$ at each nucleotide position for each sampling location (mountain) of each species. Pairwise $F_{ST}$ values were estimated, defining each sampling location as a population. SNP data was exported to PLINK format and analysed with custom R v. 2.15.1 (R. Core Team 2012) scripts.

Population structure was inferred using the software Admixture v1.3 (Alexander et al., 2009) and a PCA, which was performed with SNPSRelate (Zheng et al 2012). For both analyses, a subset of the data with loci present in at least 80% of the samples was used. Values of K ranging from one to seven in *Berberis* and one to 13 in *Juniperus* were assessed for the Admixture analysis. The value of K that exhibited the lowest cross-validation error was chosen for plotting.

*Timberline-alpine grassland distribution of glacial and interglacial periods*

The distribution of the habitat of *J. monticola* and *B. alpina* was modelled using confirmed data points of timberline-alpine grasslands of the TMVB. This “ecosystem approach” to species distribution modeling (SDM) is useful to examine the habitat
distribution of rare species across climate fluctuations (Graham et al. 2006). Although this approach has been shown to perform below average with respect to model sensitivity, it excelled in specificity statistics and robustness against extrapolations far beyond training data, suggesting that the ecosystem approach is well suited to reconstruct historical biogeography and glacial distributions (Roberts & Hamann 2012). Alpine grassland herbarium and fieldwork records were used as presence points and independent environmental variables were extracted from the 19 bioclimatic layers of Hijmans et al. (2005). The modeling was performed using Maxent v. 3.3.3k (Phillips et al. 2006) and the potential distribution of the timberline-alpine grassland was projected to the LGM using the bioclimatic layers obtained from CCSM and MIROC initiatives (Braconnot et al. 2007). Full details of the modelling are available in Supporting Information 2.

*Landscape genomics analyses*

To examine if genetic differentiation and endemism can be explained by the degree of historical spatial isolation among mountains we tested for isolation by resistance (IBR) vs isolation by distance (IBD). The approach is similar to studies where the influence of landscape features on population structure is investigated (e.g. McRae et al. 2008; Moore et al. 2011).

Resistance distances (McRae 2006) for the IBR tests were used to estimate the effective distance among sampling localities, using as a conductance grid (the reciprocal of the resistance) each of the 13 resistance surfaces described below. This method is based on circuit theory and considers multiple potential paths of least resistance between sampling points (McRae 2006), thus performing better than similar approaches such as least-cost path analysis (McRae & Beier 2007; Moore et al. 2011).

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Mantel tests with 10,000 permutations were performed to test for IBR and IBD using the pairwise effective distances for each resistance surface and the pairwise $F_{ST}$ matrices of genetic differentiation for each species. The genetic differentiation matrices were linearized using the formula for isolation by distance $F_{ST}/(1 - F_{ST})$ as advocated by Rousset (1997). Subsequently, partial Mantel tests with 1,000 permutations were performed partialling out the flat resistance distances. Tests were carried out independently for both species and also for a subset of $J. monticola$ populations (excluding Nevado de Colima and Tancitario, see discussion). Analyses and graphical representations of data were performed with R using the packages ape (Paradis et al. 2004), vegan (Oksanen et al. 2016), ade4 (Dray & Dufour, 2007) and ggplot2 (Wickham & Chang 2013).

The 13 resistance surfaces used here were based on: (i) environmental modelling (“present”, “CCSM” and “MIROC” for the LGM); (ii) a “flat” landscape, and; (iii) elevation data (above 1800, 2000, 2300, 2500, 2700, 3000, 3300, 3500 and 4000 masl; Fig. 2). The ‘flat’ landscape surface is equivalent to testing for IBD using Euclidean distances, but it takes into account the fact that the underlying landscape is bounded and not infinite (Lee-Yaw et al. 2009; Moore et al. 2011). Resistance distances were estimated using the pairwise mode of the program Circuitscape v. 3.5.8 (McRae 2006; McRae & Beier 2007) setting the sampling locations as focal points. See Supporting Information 2 for further details. The suitable area in a 30 km radius was measured for each mountain for the ccsm, present, 3000 and 4000 resistance surfaces.

Comparative demographic inference using the aggregate site frequency spectrum

We tested which demographic syndrome (population expansion, contraction or constant size) best fits the history of each sampled mountain locality for both species, and then examined if the inferred demographic changes were synchronous. Each
mountain was considered an independent population with the aim of examining the role of the mountains themselves on co-demography through time.

We used the program Multi-DICE for comparative co-demographic inference of independent populations within a unified analysis (Xue & Hickerson, 2017). This method exploits the aggregate site frequency spectrum (aSFS), which is a summary statistic vector that contains signal of co-demographic population size changes and is assembled via a re-ordering procedure applied to genomic-scale data across independent populations in the form of the site frequency spectrum (SFS) (Xue and Hickerson, 2015; see Prates et al 2016 for an applied example). The aSFS can be coupled with an inferential framework to compare observed data with simulations generated under a hierarchical co-demographic model that freely hyperparameterizes degree of synchronicity (Xue & Hickerson, 2015, 2017, Prates et al 2016). Three main steps were applied to perform this analysis:

a. Downprojecting SNP data to equal sampling size. Since the aSFS assumes independence among populations as well as all requiring populations to be sampled for the same number of individuals, we re-performed SNP calling on each population individually, which minimized the amount of missing data and thus maximized the number of SNPs within each population (Xue and Hickerson 2015, 2017; Prates et al. 2016). Since there exists a trade-off among SFS sampling projections between more individuals and less applicable populations, as well as accommodating a higher number of sampled individuals versus a lower number of SNPs due to missing data (though the opposite relationship is the case with a low number of individuals due to an increase of monomorphic sites at lower sampling projections), two downprojected datasets that allow for alternative numbers of individuals per population were constructed. This was achieved by running the populations program of Stacks to export SNPs in order to keep...
loci that were present in a minimum of four and five individuals per population (-r flag), respectively. The SNP data of each population were then down-projected using dadi 1.7 (Gutenkunst et al. 2009) to an SFS with sampling size of four and five, respectively.

b. Performing independent single population demographic analyses. Fastsimcoal version 2.5 (Excoffier et al. 2013) with the FREQ setting was used to directly generate 100,000 folded SFS simulations per model of instantaneous expansion, instantaneous contraction, and constant size for a total of 300,000 simulations, per each of the two down-projection datasets. Each SFS was simulated under 1,000 genealogies, which is based on the average number of SNPs across all the empirical SFS, and the following priors: time of size change $\tau \sim U(1,000, 250,000)$ generations ago; magnitude of size change $\epsilon \sim U(0.02, 0.20)$ for expansion model and $\sim 1/U(0.02, 0.20)$ for contraction model; effective population size $N_e \sim U(100,000, 2,000,000)$ for expansion model, $\sim U(50,000, 500,000)$ for constant size model and $\sim U(5,000, 100,000)$ for contraction model. To clarify, these distributions were selected in the interest of discriminating general demographic syndromes as well as broadly encompassing all populations within the dataset, particularly for the downstream unified comparative analysis employing hierarchical co-demographic modeling (see following step c.), and not necessarily for thorough parameter estimation per every population. Following the simulations an approximate Bayesian computation (ABC) was done using the R package abc (Csilléry et al. 2012) o to infer demographic syndrome, as well as to estimate posterior distributions for time, magnitude of population size change and effective population size. Median and mode statistics of posterior distributions were calculated for point estimates. Simple rejection was performed with a tolerance level of 0.005.
c. Unified comparative analysis based on the aSFS. The results of the single population analyses were used to inform a multi-population co-demographic analysis, for which there were 10 expanders and four contractors (total of $n = 14$ populations) for the data down-projected to four individuals, and eight expanders and four contractors (total of $n = 12$ populations) for the data down-projected to five individuals. To clarify, a single analysis was performed per data projection that combined expanders together with contractors, as there is no strong indication that these demographic trajectories were in response to differing environmental conditions, either from the single population results nor from existing literature. Additionally, the much lower number of contractors would result in drastically reduced accuracy as well as utility in estimating hyperparameter values with the aSFS if analyses were categorised by demographic syndrome.

The priors for the SFS simulation were set to: time of size change ~ $U\{100,000, 2,000,000\}$ generations ago with a pulse buffer on the prior $\beta = 20,000$ generations (forcing all other time draws to be > 20,000 generations apart from each synchronous pulse timing; Xue & Hickerson, 2017), magnitude of size change ~ $U(0.05, 0.20)$ for expansion model and ~ $1/U(0.05, 0.20)$ for contraction model, and effective population size ~ $U\{50,000, 500,000\}$ for expansion model and ~ $U\{5,000, 100,000\}$ for contraction model. Notably, the range for the time prior here is significantly widened with respect to the single population analysis in the interest of accommodating a wide range of potential co-demographic temporal scenarios (i.e. from idiosyncrasy to synchrony). Importantly, both $B.\text{alpina}$ and $J.\text{monticola}$ have a reproductive system of overlapping generations coupled with long lived reproductive individuals (Peterson 2003; Francis 2004; Adams 2008; Bonner 2008), compromising the accuracy for absolute estimation of time. While this could be addressed with translating number of generations into time.
via weighing individuals by their reproductive value (Felsenstein 1971), this is not feasible for our species. Furthermore, without reliable information regarding mutation rate nor effective population size, there is no solid foundation for calibrating the time parameter (Xue and Hickerson 2017). However, given that each population experiences comparable habitat conditions (i.e. sky islands of approximately equivalent size) as well as belongs to one of two species with similar life histories and growth forms, it is not unreasonable to assume that the same effective population size prior can be applied universally across all populations. Therefore, time estimates, which are based on effective population size values within this coalescent framework, are directly comparable and in turn hyperparameter inference can be achieved.

Per dataset (i.e. down-projections to four and five individuals), two reference tables were constructed, each with a different hyperparameterization scheme. Following Xue & Hickerson (2017), the two hyperparameterization schemes included: (1) restricting synchrony to only a single pulse and varying the proportion of taxa having membership within this pulse $\zeta \sim U\{1, n\}/n$, with the remaining taxa temporally idiosyncratic in size change (the case of $\zeta = 1/n$ would represent total idiosyncrasy); (2) distributing taxa equally across synchronous pulses, the number of which is varied $\psi \sim U\{0, 3\}$, thus allowing no idiosyncratic taxa except in the case of total idiosyncrasy ($\psi = 0$). To clarify, for the latter scenario, when $n$ is not divisible by the number of pulses, the remainder is distributed randomly across as many pulses as possible (e.g. if $n = 14$ and $\psi = 3$, then a random two of the three pulses would have five taxa and the remaining would have four taxa). This model simplification allows more accuracy in searching hyperparameter space while still providing co-demographic insight that supplements $\zeta$ inference, following the strategy outlined in Xue and Hickerson (2017). To construct each of the four total reference tables, 1,000,000 folded aSFS were simulated, with each
aSFS partitioned between the expanders and contractors such that the SFS simulated under expansion and the SFS simulated under contraction were each separately converted to two aSFS vectors, which were then concatenated (Prates et al. 2016; Xue & Hickerson, 2017). Each per-population SFS was simulated under 1000 genealogies using the priors mentioned before.

Hierarchical Random Forest (hRF) was performed for each reference table using the R package randomForest (Liaw & Wiener, 2002) and hierarchical ABC (hABC) was performed using the R package abc. To conduct hRF, we used 100 iterations of randomly selecting 5,000 simulations from the reference table to produce 10 decision trees, with the default 33% of variables per decision tree node. Decision trees were built to capture variation in $\zeta$ for the first hyperparameterization scheme and $\psi$ for the second hyperparameterization scheme, and exploited for prediction of respective hyperparameter values from the empirical data using the R function predict(). To conduct hABC, simple rejection was performed with a tolerance level of 0.0015. The function abc() was deployed for hyperparameter estimation and parameter summary estimation of dispersion index (variance/mean) $\Omega$ and mean for timings of demographic changes across populations (with lower values signaling greater synchronicity). Median and mode statistics of posterior distributions were calculated for point estimates. For both hRF and hABC, "leave-one-out" cross-validation, which involves removing a simulation and treating it as a pseudo-observed dataset (POD) for estimation against the remaining reference table, was performed using the function cv4abc following the same specifications with 50 total PODs per reference table. Estimated values were leveraged against true POD values to calculate Pearson’s correlation $r$ and root mean squared error.
Results

Alpine grassland distribution during glacial/interglacial stages

The uncorrelated environmental variables selected for the timberline-alpine grassland modelling were isothermality, mean temperature annual range, temperature in the wettest quarter, precipitation seasonality and precipitation in the coldest quarter (Fig. 3a). For the present conditions (Fig. 3b), our modelling is congruent with the known distribution of the timberline-alpine grasslands in this region (Rzedowski 1978; Calderón de Rzedowski & Rzedowski 2005), but it may represent a slight overestimate (see Supporting Information 2 for discussion). The projection to the LGM shows that this ecosystem occurred in the same geographic areas, but with a larger distribution extending to lower elevations (Fig. 3b).

RAD-seq data yield

Berberis data used here correspond to the subset of “putative orthologs within B. alpina” described in Mastretta-Yanes et al. (2014). The dataset contains 3,669 SNPs (considering only the first SNP of each RAD-locus) with an error rate of 2.3% (SD 0.27), 19% missing data and a mean coverage of 10.3 (SD 4.6). For the Juniperus data (only J. monticola ingroup), 2,925 SNPs (considering only the first SNP of each RAD-locus) were recovered, with a SNP error rate of 1.4% (SD 0.8), 16% of missing data and a mean coverage of 84.60 (SD 50.06).

Population genomic statistics and population differentiation

Populations from both species show private alleles ranging from 154 to 1101 and an average nucleotide diversity (π) ranging from 0.08 to 0.13 (Table 1). FIS decreases from east to west for Berberis and shows no clear pattern in Juniperus, with values for FIS from
0.03 to 0.07 (Table 1). Pairwise $F_{ST}$ values for *B. alpina* populations ranged from 0.056 to 0.123 and all comparisons were significant, with the Cofre de Perote population showing the highest levels of differentiation and Tlaloc the smallest, relative to other populations (Table S2.1). For *J. monticola* pairwise $F_{ST}$ ranged from 0.022 to 0.074 and all comparisons were significant, with La Malinche population showing the highest values of differentiation relative to other populations, and Tlaloc the smallest (Table S2.2).

The Admixture analyses of *B. alpina* returned a value of six as most optimal for K (Fig. S2.2a). The six groups broadly correspond to each mountain, but with some admixture between the westernmost populations (To-Aj) and among the central populations (Aj, Iz, Tl). The first four components of the PCA (Fig. S2.3a) explain 42.7% of the variance and reflect the same structure as the Admixture analysis. *Juniperus monticola* showed less structure, with K=3 (Fig. S2.2b) and 16.7% of the variance explained (Fig. S2.3b). Individuals from the west (Co, Ta) and east (Ci, Ne, Pe) form two groups that present some admixture with a third group composed of the remaining populations. Admixture within the third group is most pronounced within its eastern (Ma) and western (Bl) extremes.

*Isolation by resistance*

The resistance surface plots reveal that although most sampling locations are separated by comparable horizontal distances, there are important differences regarding the connectivity among locations, and these depend upon the elevation or distribution model used to set the conductance values (Fig. 2).
The Mantel and partial Mantel tests yielded positive significant results with different explanatory power depending on the surface used. The ‘flat’ landscape (i.e. IBD) was outperformed by some of the scenarios considering the environmental modelling or the elevation grids (Table 2). For \textit{B. alpina} the highest explanatory power was provided by the resistance surface of 3,000 masl both for the Mantel ($r = 0.940, p < 0.01$; Table 2), and the partial Mantel test ($r = 0.717, p < 0.05$). For \textit{J. monticola}, considering all populations, the surface with the highest explanatory power was the flat surface (Mantel $r = 0.499, p < 0.05$) and no partial Mantel test was significant. However, when excluding the populations of Nevado de Colima and Tancítaro, environmental modelling for the LGM using the CCSM layers provided the highest explanatory power both for the Mantel ($r = 0.686, p < 0.001$) and partial Mantel test ($r = 0.453, p < 0.01$).

\textit{Comparative demographic inferences using the aggregate site frequency spectrum}

A high degree of temporal synchrony among population size changes in both species was detected across both single population and multi-population analyses and both sampling levels. The single population analyses (Table S2.3) showed that 10 populations are consistent with population expansion (\textit{Berberis Aj}, Iz, Ma, Ti, \textit{Juniperus Aj}, Bl, Ch, Iz, Ne, Ti; population codes as in Fig. 1, see Supplementary Materials 2 for details on each population inference), four are consistent with contraction (\textit{Berberis To}, \textit{Juniperus Co}, Ma, To), two are consistent with constant size (\textit{Juniperus Ta}, \textit{Berberis Pe}) and two are inconclusive though largely consistent with constant size (\textit{Juniperus Ci}, Pp). The estimates for the respective times of expansions and contractions (Table S2.3) appeared qualitatively similar overall (expansions median = 183,549-203,767 and mode = 224,859-232,145 generations ago; contractions median = 163,888-182,777 and mode = 215,792-228,586 generations ago) and the magnitudes (ancient/present...
population size; Table S2.3) of size change were moderate as well as relatively similar in value across both population expansions (median = 0.115-0.126, mode = 0.139-0.178) and contractions value (median = 8.749-10.537, mode = 8.749-10.537; consider that the factor of expansion is the inverse of the magnitude). For the aSFS-based co-demographic analysis, across both hRF and hABC approaches and both sampling down-projections, there was an inference of low variability in the distribution of expansion and contraction times based on the $\Omega$ estimates (median $\Omega$ for uniform $\zeta$ distribution = 28,888 and 52,317, for four and five individuals, respectively) as well as a high proportion of populations within a single synchronous co-demographic pulse (hABC median $\zeta$ = 0.929 and 0.917, for four and five individuals, respectively), indicating a high degree of synchronicity (Tables 3, S2.4-5).

Discussion

We tested for population genetic differentiation and habitat persistence within the TMVB, by coupling species distribution modelling for glacial/interglacial cycles, landscape genomic analyses with explicit quantitative hypotheses, and analyses of demographic history within a comparative framework. These complementary sources of evidence support the hypothesis that tropical mountains have facilitated the differentiation and in situ persistence of alpine-grasslands species from the TVMB during the climate fluctuations of the Pleistocene.

Altitudinal changes of alpine grasslands

Ecosystem distribution modelling reveals that the alpine grasslands shifted altitude during the Pleistocene climate fluctuations, but would have persisted within some mountains during both glacial and interglacial periods (Fig. 3). It is important to note
that both our modelling approach and the available palynological and geological data are not species specific, and taxa may respond differently to subtle environmental differences or have different tolerance thresholds (Araújo & Guisan 2006; Roberts & Hamann 2012). Nonetheless, broadly speaking, the present and past distributions of timberline-alpine taxa from the TMVB are highly dependent on temperature or temperature associated variables, which in turn are highly correlated to altitude (Beaman 1962; Lauer 1978; Almeida-Leñero, et al. 2007). Thus, it is expected that the altitude of the landscape separating the highest peaks of the TMVB would play a key role in population isolation.

The modelled lower elevations of TMVB timberline-alpine grasslands during the LGM (Fig. 3c) is congruent with fossil pollen records down to 2,300-2,500 masl for reduced forests (similar to open forests close to the timberline) and grasslands (Lozano-García & Ortega-Guerrero 1994, 1998; Lozano-García et al. 2005; Caballero-Rodríguez et al. 2017). Moraines also reveal that snow lines dropped by approximately 1,000 m during glacial periods (Lozano-García & Vázquez-Selem 2005; Vázquez-Selem & Heine 2011). Considered together, the palynological, geological and niche modelling data all suggest that open forests and grasslands could have extended down to 2,300-2,500 masl at the LGM, and that suitable conditions for alpine vegetation (now at around 4,000 masl) could have been present at 3,000-3,300 masl.

Within the higher stratovolcanoes that reach more than 3,500 masl, altitudinal shifts of approximately 1,000 m can be achieved within a relatively short horizontal distance (3-6 km). This means that the alpine grasslands will shift altitudinally during glacial and interglacial periods, but with only limited horizontal displacement in the highest mountains. When such mountains are flanked by lower altitude terrains, forests
are expected to both remain isolated and persistent locally over timescales exceeding the periodicity of a glacial cycle (Fig. 3b-c).

Glacial distribution explains population differentiation

Populations of both *J. monticola* and *B. alpina* are genetically structured. Structure is more marked in *Berberis*, which could be expected given the limited number of highly isolated mountains where it was found. However, in both species the western and eastern populations are more differentiated than populations within the central part of their distributions, which presented more admixture (Figs. S2.2 and S2.3). These central populations correspond to the part of the TMVB where topography facilitates connectivity among the sampled mountains (Fig. 2), suggesting a role for landscape driving population differentiation.

Testing for IBR using surfaces that consider present and past potential habitat distributions shows that, as predicted, accounting for topography-driven connectivity better explains population differentiation than simple geographic distance (Table 2). The population genetic differentiation of both species was better explained by resistance surfaces likely representing their glacial distributions (~1,000 m below the elevation where they are currently more abundant). This result is not surprising when considering that: (1) the timberline attained its present altitude only 3,000 yr ago (Lozano-García & Vázquez-Selem 2005); (2) the last 700,000 yr have been dominated by major glacial periods with a ~100,000 yr cycle interrupted by relatively short warm interglacials (Webb & Bartlein 1992); so that (3) recent distributions could be considered a perturbation of the “historical average”, and (4) *Berberis* and *Juniperus* are slow growing and long lived, such that the number of generations representing the present distribution could be relatively small.
For *B. alpina*, the explanatory power was highest in the IBR test with the surface allowing for connectivity at 3,000 masl both in the Mantel test ($r = 0.940$) and the partial Mantel test ($r = 0.717$, $p < 0.05$). This indicates that although simple geographic distance provides explanatory power, more of the variance is explained if connectivity through time is considered (Fig. 4). This also holds for *J. monticola*, when Nevado de Colima and Tancítaro populations were excluded from the analysis (Fig. 5), as the IBR test with the LGM-CCSM surface held more explanatory power (Mantel $r = 0.686$, $p < 0.001$, and partial Mantel $r = 0.452$, $p < 0.01$; Table 2) than the other surfaces. The Nevado de Colima and Tancítaro mountains are considerably further away from the remaining high mountains of the TMVB (Fig. 1). Importantly however, our models infer that they were not connected by alpine grasslands to the Central TMVB during the Pleistocene glaciations. They remained isolated in both LGM models, even when allowing connectivity at altitudes as low as 2,300 masl (Fig. 2). These populations were thus more likely to have been founded by long distance colonisation, as opposed to climate mediated gene flow with other populations.

Results for both species are consistent with population differentiation being influenced by the landscape matrix among mountain peaks, and historical habitat connectivity patterns associated with this. In particular, the connectivity that occurred during the likely glacial distribution of each species. This fits the prediction of gene flow occurring at higher rates during glacial periods. The importance of the topographic matrix connecting mountains is noteworthy, as even during their glacial extension species seem to have maintained a fragmented (island-like) distribution (Fig. 2). Therefore glacial admixture is expected to have occurred more readily among certain population clusters (e.g. Tláloc-Iztaccihuatl-Popocatepetl, Fig. 2), while other populations should have remained isolated during interglacial stages. This is also
supported by the Admixture (Fig. S2.2) and PCA (Fig. S2.3) analyses that reveal distinct groups for the more topographically isolated populations, and more shared ancestry and gene flow among populations inferred to have higher connectivity during the glacial stages (e.g. Aj, Pp, Iz, Tl, Fig. 2).

Population differentiation and persistence under a sky-island dynamic

Our analyses of historical habitat distribution, together with genetic differentiation and co-demographic history of two high altitude plant species, support a sky-island dynamic within the TMVB that has promoted population differentiation and long-term in-situ persistence. Niche modelling demonstrates that since their emergence during the last 1.5 Myr (Ferrari et al. 2012), the highest volcanoes of the TMVB have provided stable conditions throughout glacial-interglacial cycles suitable for continuous population persistence for subalpine and alpine taxa.

The genetic data supports a scenario of long-term population persistence in both species. Genomic differentiation was significant among all populations, with $F_{ST}$ values typically greater than 0.05 (Table S2.1 and S2.2), which is congruent with populations diverging in allele frequencies and accumulating private alleles through the effects of genetic drift and mutation, and reduced amounts of gene flow (Table 1). Geographic structuring of genetic variation was found for both species (Fig. S2.2 and S2.3), with genetic groups matching the levels of glacial connectivity expected from topographic variables.

In addition to differentiation, a demographic syndrome of either moderate (expansion and contraction magnitudes ~ 9x) population expansion or constant size was inferred for most populations of both species (Table S2.3). These magnitudes are close to the lower end of the prior used (5), which is as far as low that can be detected.
by this analysis. Therefore, although some demographic change was detected, this is close to population size constancy or limited size fluctuations. This demographic history of relative stability is consistent with a sky-islands scenario for the TVBM for two reasons. First, suitable habitat is expected to have persisted in situ within the TMVB for both focal species during climate fluctuations (Fig. 3), thus allowing for in situ population persistence. Second, estimated glacial ranges are not much larger than interglacial ranges, which predicts only limited population size change through a glacial cycle. For instance, the ccsn suitable area is around 1x times larger than the present area, and the suitable area above 3,000 masl is 12x times larger than the suitable area above 4,000 (Fig. 2). More substantial demographic change is expected for species from lower altitudes of the Mexican highlands (Mastretta-Yanes et al. 2015a), and for taxa with more northern distributions (Hewitt 1996; Stewart et al. 2010), where species contracted or expanded across large horizontal distances (e.g. continental Europe) during the Last Glacial Maxima to the present.

Although we could not estimate the absolute timing of these demographic events, we can infer that they occurred synchronously among populations of both species (Table 3). Importantly, our co-demographic model did not account for genetic variation from shared ancestry or migration, which could have potentially contributed to the detected signal if such events coincided with the inferred synchronous co-demographic size changes (Xue and Hickerson 2017). Given the relatively old time of synchronous pulse (~300,000 generations ago) inferred by the aSFS analyses, the demographic signal detected could be related to the founding event following sky-islands colonization. But if such were the case, it would imply that no demographic
change strong enough to erase this signal occurred since then, which is consistent with a co-demographic history of relative population stability and persistence within sky islands.

**Conservation and management implications**

Most of the sampled mountains of this study are Natural Protected Areas (NPA) currently under different threats and management methods. Our results highlight the conservation value of the TMVB peaks, showing that they are areas of long-term biodiversity persistence despite historical climate fluctuations. Our results are also relevant for the management of these NPA. Firstly, because we show that these mountain peaks behave like islands showing high levels of genetic isolation. As a consequence, we suggest that these mountains should be managed like islands, for instance promoting the use of native germplasm for reforestation efforts. Secondly, our results show that alpine grasslands from the TMVB are a natural ecosystem that has historically persisted within these mountains. Therefore the aorestation of these grasslands, as currently done in some of the mountains, is destroying a natural ecosystem of conservation importance.

**Conclusion**

We have shown with different sources of evidence that: (1) the highest stratovolcanoes of the TMVB facilitated the existence of timberline-alpine grasslands throughout glacial/interglacial cycles (long-term *in situ* population persistence); and (2) population genetic differentiation of species from this ecosystem can be explained by the degree of habitat connectivity among mountains during the glacial periods.
Similar scenarios have been postulated for taxa of the TMVB from lower altitudes using classical population genetic and phylogeographic approaches (e.g. McCormack et al. 2008; Bryson et al. 2011, 2012; Gutiérrez-Rodríguez et al. 2011; Ornelas et al. 2013; Parra-Olea et al. 2012). Some of these previous phylogeographic studies focusing on divergence times (e.g. Ornelas et al. 2010; Bryson et al. 2012a; b; Leaché et al. 2013) have not been able to distinguish between the confounding effects of climate and geological change, because in the TMVB climate fluctuations and volcanic changes co-occurred during the Pleistocene (Mastretta-Yanes et al. 2015). However, here we assessed present versus past historical connectivity quantitatively and in a landscape explicit context. This spatial approach allows to relate population differentiation to the Pleistocene glacial cycles and the sky-island dynamics they produce in tropical mountains.

Our results support the ongoing role of tropical mountains as cradles for biodiversity by uncovering cryptic differentiation and limits to gene flow, and as museums for biodiversity by promoting long-term in situ persistence. Therefore, the conservation importance of tropical mountains, such as the ones of the TMVB, resides not only on its species richness per se, but on that landscapes like these promote both long-term population survival and further diversification.

Acknowledgments
Part of the analyses were carried out on the High Performance Computing Cluster supported by the Research and Specialist Computing Support service at UEA and at the City University of New York High Performance Computing Center, with support from the National Science Foundation (CNS-0855217 and CNS-0958379). Field work was possible thanks to Oscar Trejo from DGFS and CONANP staff from the natural protected
areas Cofre de Perote, Pico de Orizaba, La Malinche, Izta-Popo, Nevado de Toluca, Mariposa Monarca, Pico de Tancítaro and Nevado de Colima. We are thankful to SMG, JRPPK, JJRL, AOM, ROF, SSF, RAF, TSA, JAA, FDRG, FQB and MJLF for fieldwork assistance, to T. Wyss, A. Brelsford and C. Berney for laboratory work assistance and to Michael J. Hickerson and his lab for support and resources. We acknowledge the international modelling groups for providing their data for analysis, the Laboratoire des Sciences du Climat et de l’Environnement (LSCE) for collecting and archiving the model data. This work was supported by Consejo Nacional de Ciencia y Tecnología (grant number CONACYT 213538 to AMY and CONACYT 178245 to DP), by Rosemary Grant Award for Graduate Student Research from the Society for the Study of Evolution granted to AMY, by grants from National Science Foundation (DEB-1253710 to M.J.H.; DEB-1343578 to Ana C. Carnaval, M.J.H., and Kyle C. McDonald); FAPESP (BIOTA, 2013/50297-0 to A.C.C., M.J.H., and K.C.M.); and NASA through the Dimensions of Biodiversity Program.

References

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**Data Accessibility**

This project has been deposited at Dryad Repository under the accession doi:10.5061/dryad.f7248. The repository includes lab protocol, species occurrence data, spatial data, genetic data in plink format and the scripts used for all analyses. Raw RADseq data is at the Sequence Read Archive (SRA) accession SRP035472 for *Berberis* and SRP125588 for *Juniperus*.

**Author Contributions**

AMY, DP, THJ and BCE conceived and designed the study. AMY performed fieldwork and laboratory work, assembled the RAD data and made all analyses, except for the aSFS and the ecosystem distribution modelling. ATX performed the aSFS analyses. AML

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performed the ecosystem distribution modelling. NA and THJ supervised lab work and preliminary analyses. AMY, DP and BCE lead manuscript writing. All authors contributed to the discussion and manuscript writing.

Table 1. Summary population genetic statistics for *B. alpina* and *J. monticola*

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<th>(H_{obs})</th>
<th>(\pi)</th>
<th>(F_{Is})</th>
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Results include only nucleotide positions that are polymorphic in at least one population. The first column shows the number of individuals per population that were used for the analysis (Ns). Next are the average number of individuals genotyped at each locus (N), the number of variable sites unique to each population (i.e. private alleles, Priv.), the number of polymorphic nucleotide sites for that population (Sites), the average frequency of the major allele (P), the average observed heterozygosity per locus ($H_{obs}$), the average nucleotide diversity ($\pi$), and the average Wright’s inbreeding coefficient ($F_{IS}$). Populations are ordered East to West, top to bottom. Population IDs as in Fig. 1.

Table 2. Isolation by resistance

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<th>$B.\text{ alpina}$</th>
<th>$J.\text{ monticola}$ all pops.</th>
<th>$J.\text{ monticola}$ excluding Co &amp; Ta</th>
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<td>miroc</td>
<td>0.792</td>
<td>** 0.218</td>
<td>NS 0.430</td>
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<td>flat</td>
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<td>4,000</td>
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<td>* -0.017</td>
<td>NS 0.378</td>
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</table>

Associations between genetic differentiation (linearized $F_{ST}$, see main text) and pairwise effective distances at different surfaces (see Fig. 2 for an explanation of these). Mantel test $r$ value ($r$) and Partial Mantel test $r$ value (Pt $r$) are reported for each species. Significance codes are as follows: < 0.001 ‘***’, <0.01 ‘**’, < 0.05 ‘*’, and not significant ‘NS’. Underlined cells correspond to the surface with the highest prediction value for each taxon.
Table 3. Proportion of populations in synchrony and number of synchronous pulses.

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<th>Estimation method</th>
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<td>hABC Hyperparameter Estimation - Median</td>
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<td>hABC Hyperparameter Estimation - Mode</td>
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Results for the downprojection to four individuals, see Table S2.4 for results with the downprojection to five. $\zeta$: proportion of populations in synchrony (considering both species together). $\psi$: number of synchronous pulses. For $\zeta$ and $\psi$, the three columns show the resulting estimation (res), and the cross-validation accuracy check based on the correlation ($r$) and the root mean squared error ($rmse$) between the simulated true values and the estimated values.
bio1: Annual mean temperature
bio2: Mean diurnal range
bio3: Isothermality (bio2/bio7)*
bio4: Temperature seasonality
bio5: Max. temp. warmest quarter
bio6: Min. temp. coldest month
bio7: Temperature annual range*
bio8: Mean Temp. wettest quarter*
bio9: Mean temp. driest quarter
bio10: Mean temp. warmest quarter
bio11: Mean temp. coldest quarter
bio12: Annual precipitation
bio13: Precipitation wettest month
bio14: Precipitation driest month
bio15: Precipitation seasonality*
bio16: Precipitation wettest quarter
bio17: Precipitation driest quarter
bio18: Precipitation warmest quarter
bio19: Precipitation coldest quarter*